12 Amine Oxidases and Reductases

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12.1 SUMMARY

In the drug and foreign compound metabolism literature, amine oxidation and carbon oxidation reactions are well documented for the reactions of the FAD-containing monooxygenase [1] and the cytochrome P450s [2]. However, there are many other enzyme systems that are capable of catalyzing these reactions. This review provides an overview of the various amine oxidases, such as the copper-containing amine oxidases (CAOs) and flavin-containing amine oxidases. The copper-containing enzymes also have a prosthetic group formed from a tyrosyl residue in a self-catalyzed oxidation reaction with copper. There are four different prosthetic groups that can be formed from tyrosine or tryptophan and that are critical for the various catalytically important reactions of these related proteins. The flavin-containing amine oxidases play important roles in the metabolism of neurogenic amines, D- and L-amino acids and polyamines. However, they metabolize exogenous compounds as well. A second set of enzymes are involved in the reduction of azo and nitro compounds, mainly involving bacterial and mammalian flavoproteins and the cytochrome P450s. These enzyme systems also reduce quinones, halogenated compounds, hydroperoxides, and α, β-unsaturated aldehydes. This chapter describes the biochemical and molecular details of these genes

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and their gene products, with an eye to identifying tools that would be valuable in establishing which might be involved in oxidative and reductive metabolism of new molecular entities synthesized for drug therapy.

12.2 INTRODUCTION

Among the many reactions involved in the metabolism of drugs and foreign compounds, the amine oxidation and reduction reactions are mechanistically the least well documented in the literature [2]. This is due in part to the common use of a single isolated cell types or tissue fractions (microsomes, nuclei, mitochondria, or cytosol) to study foreign compound metabolism in academe and industry. Amine oxidation and reduction often are missed unless intact cells or tissue are used and a careful tissue fractionation study is performed with any new compound of interest [3]. Often, this is due to two different enzyme systems contributing to the activity observed in the same tissue fractions/cell or the second enzyme system existing in an organelle other than the microsomal fraction. Thus, such studies result in an underestimation of the role of these interesting enzymes in the metabolism of nitrogen-containing organic compounds. For example, in our early studies on hydrazine metabolism, we discovered an enzyme activity in the mitochondrial fraction of rat liver that oxidized procarbazine, a 1, 2-disubstituted hydrazine, to its azo derivative [4]. We performed careful subcellular fractionation of rat liver to demonstrate the organelle the enzyme activity was associated with (i.e., microsomes or mitochondria) and further characterized the biochemical nature of the mitochondrial activity. After the use of monoamine oxidase (MAO) inhibitors and partial purification following kynuramine deamination activity, we determined that the enzyme responsible for this activity was mitochondrial MAO. We also demonstrated that procarbazine was a competitive substrate with kynuramine. While cytochrome P450 can oxidize nitrogenous compounds such as primary and secondary amines and hydrazines [5–7], other enzyme systems also oxidize these compounds to *N*-hydroxy or azo compounds. Many of these are the MAOs and diamine oxidases (DAOs) that are discussed in this chapter.

The reduction reactions of azo and nitro compounds are reactions that also have been shown to be catalyzed by cytochrome P450s and other electron-transfer proteins, such as NADPH:cytochrome P450 reductase and NAD(P)H:quinone oxidoreductase. These reactions are also underappreciated and can account for reduction of azo, nitro, and α, β-unsaturated alkene compounds, such as neoprotosil, nitrofuran, and 4-hydroxynonenal, respectively. The work of Levine and Lu [8] demonstrated that specific cytochrome P450s, such as CYP4A and CYP3A4, account for the major catalysts for azo reduction in microsomal fractions from liver and intestine, in addition to flavoproteins, such as NADPH:cytochrome P450 oxidoreductase (POR).

12.3 AMINE OXIDASES

There are two classes of amine oxidases, those that use covalently bound quinones and copper as cofactors and those that use a prosthetic flavin group for catalysis. In general, they catalyze the same chemical reaction, namely, oxidative deamination leading to formation of an aldehyde, ammonia, and hydrogen peroxide (Eq. 12.1). All

three products formed are chemically reactive and specific enzymes are required to dispose of the reactive products formed.

$$RCH_2{-}NH_2 + O_2 + H_2O \longrightarrow R{-}CHO + NH_3 + H_2O_2 \quad (12.1)$$

Both classes display bi-bi ping-pong kinetics, which implies that two half reactions are involved in amine oxidation (Eqs. 12.2–12.4). The oxidant is an enzyme-bound prosthetic group that undergoes cyclic reduction/oxidation reactions and varies with each class of amine oxidase. An aldimine intermediate is formed in the oxidation step, but it is chemically unstable and decomposes to ammonia and the aldehyde product (Eq. 12.2). The following section describes the characteristics of these two classes of amine oxidases and the prosthetic groups involved in amine oxidation.

$$R{-}CH_2{-}NH_2 + \text{Oxidant} \longrightarrow RCH{=}NH + \text{Reduced oxidant} \quad (12.2)$$

$$RCH_2{=}NH + H_2O + H^+ \longrightarrow RCHO + NH_4{}^+ \quad (12.3)$$

$$\text{Reduced oxidant} + O_2 \longrightarrow \text{Oxidant} + H_2O_2 \quad (12.4)$$

12.3.1 Copper-Containing Amine Oxidases (CAOs)

The CAOs were the first quinoproteins discovered and their characteristics were described by Hauge [9] and Antony and Zatman [10,11] in the early 1960s for the bacterial enzymes glucose dehydrogenase and methanol dehydrogenase. However, the identity of the cofactor involved was not defined for some years and Anthony and Zatman [10] suggested it was a pteridine derivative. These proteins contain a covalently bound quinone formed from a tyrosine or tryptophan residue, which serves as an electrophilic group to covalently bind amines or alcohols, before their oxidation by a metal redox center, yielding hydrogen peroxide as a product. Through the study of bacteria and lower eukaryotes, a role for CAOs in the degradation of amines as a source of energy and basic nutrients was deduced. The presence of CAOs in mammalian tissues suggests that there may be other important roles for these enzymes. There are four known mammalian genes (*AOC1–4)* that are described in detail below. The cofactor for the bacterial enzymes was shown to be a pyrroloquinolone quinone (PPQ) by Salisbury *et al*. [12] and Knowles *et al*. [13], whose studies were based on the earlier work of Duine and coworkers [14]. No examples were known for such novel quinone cofactors in mammalian enzymes until the 1990s, when Hartmann and Klinman [15] suggested a quinone cofactor for bovine plasma copper-amine oxidase, first assumed to be PPQ. Since that time, a number of mammalian enzyme systems involved in amine oxidation have been described and a variety of quinone cofactors have been elucidated. These CAO quinoproteins are divided into four basic forms containing different posttranslationally modified tyrosine or tryptophan residues capable of enhancing the catalysis of oxidative metabolism of primary amines [16]. The four types of side chain-derived quinone cofactor groups that are formed

in eukaryotic organisms use posttranslational, self-driven oxidation of a tyrosine within a highly conserved sequence [Ser/Thr-X aa-X aa-Asn/Tyr (topoquinone (TPQ))-Asp/Glu-Tyr/Asn] to yield a peptide-bound 2,4,5-trihydroxy-phenyl-alanine quinone (TPQ) residue that is the critical catalytic prosthetic group on the enzyme for amine oxidation [17–19]. A second enzyme class consists of proteins containing a lysyl tyrosylquinone (LTQ) prosthetic group [20]. These oxidases have prosthetic groups that involve two critical amino acid residues, lysine and tyrosine, resulting in the formal adduction of the lysine residue to a TPQ intermediate similar to that deduced for bovine serum amine oxidase. The other oxidases have been shown to contain tryptophan-derived quinone cofactor quinoproteins [21–23], where cysteine tryptophanquinone (CTQ) and tryptophan tryptophylquinone (TTQ) residues are covalently linked to form a complex structure (Fig. 12.1).

The formation of the known quino-cofactors requires several steps, starting with copper-catalyzed hydroxylation of either tyrosine or tryptophan residues within a relatively conserved amino acid sequence to yield an *ortho*-quinone ring. The mechanism for the formation of these quinones has been studied with the purified *Hansenula polymorpha* amine oxidase by Klinman and coworkers [17,19]. In the case of TPQ in plasma amine oxidase, no further modification of the tyrosyl quinone occurs for productive catalysis. The unconjugated quinone carbonyl group at the para position allows for Schiff base formation with nucleophilic monoamine or adduction by other

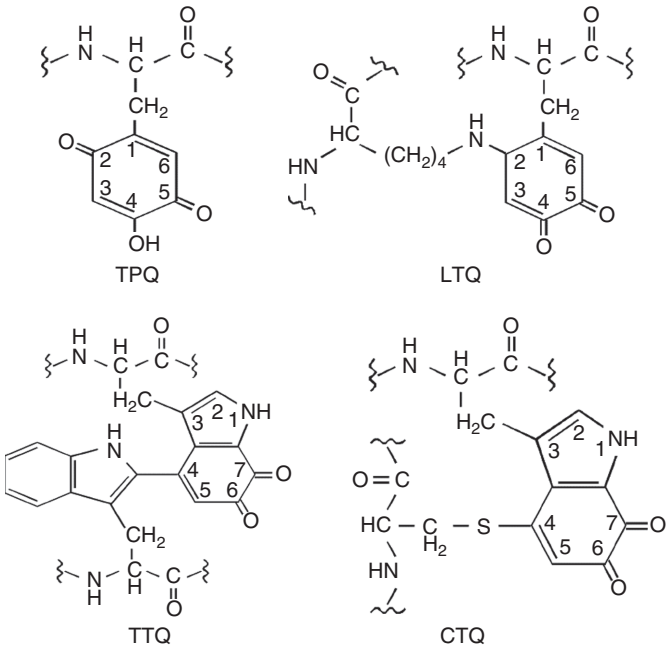

Figure 12.1 Quinone cofactors of various copper-containing amine oxidases as described by DuBois and Klinman [16]. TPQ, 2,4,5-trihydroxy-phenyl-alanine quinone; LTQ, lysine tyrosylquinone; CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone. *Source:* Modified from DuBois JL, Klinman JP. Arch Biochem Biophys 2005;433:255–265., with permission from Elsevier Inc.

inhibitory nitrogenous compounds, such as phenylhydrazine, semicarbazide, clonidine, and phenylethylhydrazine. The other quinoproteins have products of oxidized tyrosyl or tryptophyl rings cross-linked with other nucleophilic amino acid side chain residue, such as lysine, cysteine, or tryptophan. For CTQ, TTQ, and LTQ, the end products are oxidized tyrosyl or tryptophyl residues that are further cross-linked with another nucleophilic amino acid side chain residue, namely, tryptophan, lysine, or cysteine. This cross-linking process may provide a unique function for the proteins, forming a relatively stable cross-link [lysyl oxidase (LOX)], modifying the redox potential of the cofactor to enhance reactivity, protecting the reactive cofactor from side reactions, and/or reducing movement of the cofactor at the active site [24]. Further examples of such quinoproteins and the study of their reactions are needed to define the unique properties of these complex proteins. With that description of quinoproteins, we describe several classes of enzymes whose function might affect the study of drug metabolism, leading to new metabolic products not expected from the cytochrome P450s or FAD-containing monooxygenase.

12.3.1.1 Diamine Oxidase (DAO). The DAOs are a group of enzymes that display substrate specificity for oxidation of diamines, such as histamine [25]. DAO is expressed in the placenta, kidney, gut, lung, and brain. In the intestine and kidney epithelial cells, DAO is released from basolateral vesicles at the plasma membrane in response to heparin and is subsequently cleared from the plasma. The IUB nomenclature designation for DAO is EC 1.4.3.22 classifying it as an amine:oxygen oxidoreductase (deaminating) (copper) enzyme, and it is encoded by the *AOC1* gene found in nearly all mammalian species. The human enzyme appears to have a subunit molecular weight of 70–105 kDa and contains one atom each of copper and magnesium [26,27]. Histamine and 1-methyl-histamine are excellent substrates. A human intolerance syndrome in pregnancy has linked high circulating levels of histidine to decreased levels of plasma DAO [25]. Other substrates described for the human enzyme are spermine, spermidine, acylated putrescine, cadaverine, and related compounds, for example, [8-arginine]vasopressin, [8-lysine]vasopressin, collagen, and tropocollagen. Although DAO has been shown to have some activity toward monoamines, it displays little or no activity toward higher substituted amines. The pH optima of the human enzymes are in the range of 9–10, but other forms have been shown to have other pH optima.

Like the amine oxidases described below, DAOs contain copper and 2,4,5-trihydroxy-phenyl-alanine quinone. In a copper-deficient state in mammals, this enzyme activity may be diminished like the amine oxidases because it easily loses or gains copper depending on the concentration of free copper in the cell [25]. Because DAO contains TPQ and therefore a reactive carbonyl group, they are inhibited by carbonyl-specific reagents such as semicarbazide or phenylhydrazine. The enzyme also has been shown to be inhibited by drugs such as isoniazide (tuberculostatic agent), cimetidine (histamine H_2 receptor agonist), clonidine (antihypertension drug), and several antiprotozoal agents (pentamidine and berenil). 2-Hydrazinopyridine and benzylamine are inhibitors, as they are for the other CAO enzymes, because of the presence of the active carbonyl functional group of TPQ cofactor utilized in the deamination reaction.

12.3.1.2 Retina-Specific Amine Oxidase (RAO or Lysyl Oxidase, LOX). There are four LOX genes in humans (LOXL1, LOXL2, LOXL3, and LOXL4), and their full

comparison has not been fully documented to date. They are believed to be involved in the development of many tissues and also in invasive processes during cancer metastasis [28,29]. A retina-specific amine oxidase (RAO), described by Imamura *et al*. [30] as LOX, is composed of 729 amino acids giving a molecular weight of 80.6 kDa. It is encoded by the *AOC2* gene. Its IUB nomenclature designation is EC 1.4.3.13, and its substrates are collagen and elastin in the extracellular matrix (ECM) of ocular tissues. It is synthesized as a glycosylated proenzyme with molecular weight 50 kDa per subunit and is converted to its active form (molecular weight, 32 kDa) by procollagen C proteinase (bone morphogenic protein-1) before secretion to the ECM of the eye. The disparity in molecular weight may be due to the fact that it is a secreted protein and that it is heavily N- and O-glycosylated, leading to aberrant migration on SDS polyacrylamide gel electrophoresis [31]. Reports of multiple LOX isoforms can be found in the literature [31]. It is ~62.2% related in sequence to the bovine amine oxidase described by Janes *et al*. [18], and the active site is highly conserved among the amine oxidases. Imamura *et al*. [30] also demonstrated that this gene is expressed only in the eye, suggesting an important function of RAO in this tissue. Subsequent studies by Coral *et al*. [32] characterized a LOX enzyme in ocular tissues and suggested that proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RRD) result from ECM remodeling due possibly to improper collagen cross-linking. They demonstrated that LOX enzyme activity is significantly lower in the vitreous of PDR or RRD patients, relative to control subjects. This observation supports the report of Hewitt *et al*. [33] who provided a strong correlation between expression of a LOX variant in the ECM of individuals and the development of pseudoexfoliation syndrome, a condition in which the ECM appears to be aberrant and there is accumulation of abnormal fibrillar material in the anterior segment of the eye. They suggested that this syndrome may predispose humans to a glaucomatous optic neuropathy.

The roles of LOX in development and disease are numerous, as shown by the interest in its role in cancer metastasis, as well as normal tissue development [34]. It is known that strong nucleophiles (lathyrogenic agents), including nitriles, hydrazides, hydrazines, and ureides, cause defects in formation of connective tissues in developing animals. Agents such as β-aminopropionitrile, 2-chloroethylamine, and 2-bromoethylamine are also potent inhibitors of cancer cell metastasis, demonstrating the chemical nature of the quinoprotein. The critical function of LOX in processing collagen and elastin to complete the posttranslational processing of these molecules and its association with disease and defective connective tissue development provide a potentially unique future therapeutic target for future investigation.

12.3.1.3 Semicarbazide-Sensitive Amine Oxidase (SSAO). Semicarbazide-sensitive amine oxidase [SSAO; EC 1.4.3.6; (amine:oxygen oxidoreductase (deaminating) (copper-containing))] is a glycosylated dimeric copper-containing enzyme mostly bound to outer membranes of smooth muscle cells, endothelial cells, and adipocytes; it has been described as a vascular ectoenzyme [35–37] since it appears to be a secreted protein with full enzyme activity. This protein is encoded by the *AOC3* gene. It was initially discovered as a benzylamine oxidase activity found in cultured porcine aortic smooth muscle cells and smooth muscle cell homogenates [38–40]. The enzyme's activity is also present in other tissues, such as uterus, ureter, and vas deferens. Research characterizing the toxicity of allylamine on myocardium and other vascular tissues suggested that SSAO is responsible for the oxidative metabolism to

acrolein, the toxic intermediate responsible for tissue damage. The toxicity toward the myocardium was prevented by administration of hydralazine, a hydrazine-containing drug [41–44], which now can be explained by its adduction to the reactive carbonyl of the quinone cofactor. The unique toxicity of allylamine reported in the literature led Boor and coworkers to characterize the protein by following its semicarbazine-sensitive benzylamine oxidizing enzyme activity. Boor and coworkers [45,46] demonstrated that swine aortic smooth muscle cell homogenates or cultured smooth muscle cells could oxidize allylamine to acrolein and lead to the view that acrolein most likely is the toxic agent causing the deleterious effects of allylamine on myocardial and vascular dysfunction. The purified protein was shown to be a homodimer with each monomer displaying a molecular weight of 90–100 kDa when isolated from either bovine lung or swine aortic smooth muscle cells [42,45,47]. From the primary sequence of the bovine lung enzyme, it contains 762 amino acids to provide a molecular weight of 84.8 kDa. The first 16 amino acids of its N-terminus possibly represent a signal peptide involved in synthesis and secretion. The extracellular portion of SSAO is apparently cleaved to generate a soluble enzyme that circulates in the plasma [42,44], explaining why Boor and coworkers [45,46] could isolate the enzyme from the media taken from cultures of swine aortic smooth muscle cells. Although the protein was identified as a secreted extracellular protein, cell fractionation showed that the majority of intracellular SSAO activity is membrane bound with the principal intracellular site of localization in the microsomal fraction that also contain the golgi apparatus that participates in the secretion process. Careful fractionation of plasma membranes also provided evidence that SSAO is predominantly localized in the plasma membrane of rat and aorta smooth muscle cells, likely facing away from the cell membrane where it can encounter its substrates [35,42]. Since it can be found in the media during culture of swine aorta smooth muscle cells [46,48], it appears that it can be processed to a soluble form as discovered by Boor's group.

In addition to benzylamine and allylamine, circulating amines such as methylamine, 2-phenylethylamine, aminoacetone, histamine, tyramine, tryptamine, and protein-bound amines appear to be substrates for SSAO [35]. The substrate specificity varies depending on the species studied. As predicted by the reaction it catalyzes (Eqs. 12.2 and 12.3), it displays a ping-pong catalytic mechanism. A unique feature of the enzyme is that it binds copper tightly relative to the DAO and plasma amine oxidase (PAO) [35,46]. As its name implies, it is inhibited by strong nucleophiles, including semicarbazine, amiloride, hydralazine, phenelzine, and phenylhydrazine.

SSAO has been shown to be identical to vascular adhesion protein-1 (VAP-1) that is induced during inflammation and mediates interaction between lymphocytes and endothelium [49]. The tissue distribution of VAP-1 is similar to SSAO and is responsible for inducing lymphocytic cell adhesion and granulocyte extravasation [50]. Ongoing research in vascular biology suggests that baseline VAP-1/SSAO activity can be used as a predictor of hemorrhage after tissue plasminogen activator (tPA) treatment. Since semicarbazide and other compounds, such as hydralazine, ameliorate VAP-1/SSAO activity, it is proposed that anti-VAP-1/SSAO drugs given with tPA may prevent neurological disorders following with ischemic stroke [51].

12.3.1.4 Plasma Amine Oxidase. The plasma amine oxidases are the best studied mammalian CAO, as exemplified by the extensive research done by a number of groups and the seminal work of Klinman and her collaborators in identifying the cofactor [16].

Among the early publications on the plasma amine oxidases was a report by Mason and coworkers [52] demonstrating that this enzyme contains copper in its active state; the enzyme also has been suggested to contain zinc, which is essential for its activity. Yet a number of studies using electron paramagnetic resonance could not detect a redox change in the Cu(II) center during catalysis [53]. Blaschko and coworkers [54] also demonstrated the essential role of copper by administering a diet deficient in copper and measuring the resulting enzyme activity of the plasma. As a copper enzyme, its bluish color can be observed during the later steps of the purification process. In addition, Blaschko *et al*. [54] suggested the enzyme contained pyridoxyl phosphate because of its absorbance at around 440 nm, a common characteristic of the TPQ that can be followed spectrophotometrically during the course of self-catalysis of the native protein to the active posttranslational modified enzyme. Subsequently, several groups [13,54] documented that the purified enzyme did not contain pyridoxal phosphate, but on the basis of NMR spectra suggested that it contained PPQ. At the time Klinman began her studies on plasma amine oxidase, she referred to PPQ as the cofactor in her 1987 publication using reductive trapping of substrate/inhibitor to the bovine plasma amine oxidase [15]. Subsequently, she deduced that the cofactor was not PPQ, but a new quinone cofactor, TPQ. Her group also established the oxidative mechanism by which the tyrosyl residue was converted to the TPQ cofactor [17–19, 54].

Plasma amine oxidases are listed with the IUB nomenclature EC 1.4.3.21 as being amine:oxygen oxidoreductase (deaminating) (copper-containing). It is encoded by the CAO4 gene (*AOC4*) in humans [44]. These enzymes have been purified and characterized from several species (bovine, humans, swine, and pea seedlings); they share many properties in common. The PAOs display native molecular weights of 180 kDa and are composed of two subunits (70–90 kDa) accounting for the presence of two copper (II) molecules and two active carbonyl groups per mole of enzyme, the latter being susceptible to adduction with amines and hydrazines. Three critical histidine residues are proposed to be available for the binding of copper, and early work using zinc to displace copper demonstrated that this protein can easily be made replete of copper. This observation also provides an explanation for reduced levels of PAO in plasma during dietary depletion of copper [44,52,55]. Although benzylamine and methylamine have been the major substrates used in studies of this enzyme, there are reports that the natural substrates for this class of enzymes are physiologically active amines, such as spermidine and spermine, catecholamines, and tryptamine derivatives. Other amines such as tyramine have been shown to be oxidized but with low affinity, and tryptamine, epinephrine, serotonin, or agmatine do not appear to be substrates [52,55]. The pH optima for the reactions varies depending on the substrate in use; for example, for spermine, the pH optimum is 6.2, while for spermidine it is 7.2 [56].

AOC4 is known to be expressed in mammalian liver, with the encoded protein being a secreted protein, leading to the high levels seen in bovine and porcine blood. In humans and rodents, there are much lower levels of this activity in plasma, although these species all have an *AOC4*-related gene. In these species, *AOC4* exhibits a single base change that converts the codon 255 (tryptophan) to a stop codon, yielding a nonfunctional, truncated protein [44]. In humans, the mRNA can be detected in the liver, and splice variants of the gene have been reported [57]. In the mouse and rat, only fragments of the *AOC4* gene exist.

Since the enzyme is a quinoprotein, strong nucleophiles, such as aryl- and alkylhydrazines, and other nitrogenous compounds inhibit oxidation by forming hydrazide or

semicarbazide forms of the cofactor. Clonidine, an antihypertensive that binds to α_2 receptors, is an inhibitor that changes the orientation of the TPQ relative to copper, yielding an inactive cofactor–metal complex [58]. Adduction by these compounds to TPQ prevents the nucleophilic attack by the amine substrate as the first catalytic step, as shown by Klinman and DuBois [16] for phenylhydrazine.

12.3.1.5 Summary of CAOs. Since these CAOs have K_{cat} values equal to or greater than those of the cytochrome P450s, drugs and foreign compounds that contain a primary amine or that can be converted to a primary amine can be oxidatively deaminated to aldehyde metabolites. These aldehydes, such as acrolein in the case of allylamine, can cause toxicity, particularly if they are generated near sensitive target organs and have been proposed to result in aberrant cell signaling [59]. In addition, if primary amines contain an amino group that is a good nucleophile, but are not CAO substrates, they can serve as competitive inhibitors altering the disposition of other substrates. These properties allow the CAOs to be part of a drug–drug interaction mechanism that should be considered where warranted during drug development.

The CAOs are affected by dietary depletion of body stores of copper, and therefore, copper-deficient patients might experience difficulties with certain drugs or compounds that are CAO substrates, as has been seen with histamine-sensitivity syndrome seen during pregnancy. To date, relatively few studies exist related to the study of CAO regulation or limits on the self-driven posttranslational modification to form an active enzyme. Therefore, when amine compounds are a focus of study, one should be aware that CAOs are clearly involved in amine metabolism and are also critical for defined steps in collagen processing and connective tissue formation.

12.3.2 Flavin-Containing Amine Oxidases

A number of flavin-containing amine oxidases exist that rely solely on a flavin prosthetic group as the oxidizing cofactor in their catalytic mechanism (Eq. 12.2). These enzymes are distinctly different from the microsomal FAD-containing monoxygenases first described by Ziegler and Poulsen [1]. Owing to the role of flavin-containing amine oxidases in the metabolism of monoamines and D- and L-amino acids, they have been extensively studied and are defined as being amine:oxygen oxidoreductase (deaminating) enzymes with an IUB EC 1.4.3 designation. The enzyme family consists of several subclasses depending on their subcellular localization and amine substrate preferences. They are distinguished as MAOs, DAO, and polyamine oxidases. Because there is considerable sequence identity among these oxidase enzymes, it is presumed that they have similar molecular structures. In addition, there are multiple enzyme forms within each subfamily as seen below. The catalyzed reactions are a subset of the reactions as shown in Equations 12.1–12.4, except a flavin, such as FAD or flavin mononucletide (FMN), is used as the prosthetic group. The first products formed in their reaction is reduced flavin and an aldimine intermediate (Eq. 12.5) that subsequently hydrolyzes to form an aldehyde and ammonium ion (Eq. 12.6). Molecular oxygen is the terminal oxidant for most reduced flavoproteins of this group (Eq. 12.7).

$$R{-}CH_2{-}NH_2 + FAD \longrightarrow RCH_2{=}NH + FADH_2 \tag{12.5}$$

$$RCH_2{=}NH + H_2O + H^+ \longrightarrow RCHO + NH_4{}^+ \tag{12.6}$$

$$FADH_2 + O_2 \longrightarrow FAD + H_2O_2 \tag{12.7}$$

12.3.2.1 Monoamine Oxidase A (MAO A and MAO B). The MAOs are flavin-dependent oxygenases catalyzing the same basic reaction as the copper-containing quinoprotein class of enzymes (Eq. 12.1). A review article by Shih *et al*. [60] provides an overview of the MAOs and has an extensive reference list addressing the characteristics of the two enzyme forms (A and B) that are classified as EC 1.4.3.4 enzymes [61]. Their molecular weights deduced from their amino acid sequences are 59.7 and 58.0 kDa [62], respectively, and they have 70% amino acid sequence identity. MAO A and B are encoded by separate genes located on the X chromosome. They are membrane-bound enzymes found in the outer mitochondrial membrane in the brain and many other tissues, including liver and kidney [63]. Their cofactors are covalently linked FAD molecules, with a cysteine thiol linkage to the 8a position of the isoalloxazine ring [64]. The best studied substrates include dopamine, noradrenaline, 5-hydroxytryptamine, and tyramine (MAO A substrates), while benzylamine and 2-phenylethylhydrazine are preferred substrates for MAO B [60,65]. Excessive intake of tyramine, an MAO A substrate has been associated with the "cheese" reaction, in which processing of tyramine is inhibited by MAO inhibitors, leading to hypertensive crises. Clearance of some foodstuffs high in tyramine or other vasoactive amines is now known to be dependent on the MAOs, and their routine clearance is affected by the MAO inhibitors [39,66]. MAO B also oxidizes 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to its toxic metabolite. Production of this metabolite apparently is involved in the selective destruction of nigrostriatal neurons, resulting in a Parkinson-like disorder [67]. There are also form-specific inhibitors that allow differential inhibition in experimental studies, namely, clorgyline (MAO A specific) and deprenyl and pargyline (MAO B specific). Some species-specific differences in substrate specificity and inhibitor sensitivity are seen between the two enzymes.

In addition to amines, Coomes and Prough [4] demonstrated that MAO can oxidize monoalkyl- and 1,2-disubstituted hydrazines to form diazene and azo metabolites, respectively [68]. In the case of the monosubstituted hydrazines, the diazene intermediates are potent electrophiles capable of protein or nucleic acid binding, while the azo metabolites of 1,2-dimethylhydrazine and procarbazine (a 1-methyl-2-benzylhydrazine derivative) are stable but can be further oxidized to azoxy metabolites that are mutagenic [68]. Erikson and Prough [69] demonstrated that MAO catalyzed the oxidative metabolism of procarbazine, 1,2-dimethylhydrazine, *n*-propylhydrazine, and iproniazid. In the case of procarbazine, both clorgyline and propargyline were inhibitors of MAO A- and MAO B-catalyzed oxidation to azoprocarbazine. Although endogenous amine metabolism by MAOs has been extensively studied because of their role in clearance of neuroamines, their metabolism of exogenous amines has been less well characterized.

12.3.2.2 D-Amino Acid Oxidases (DAAO). The pig kidney D-amino acid oxidase (DAAO) was one of the earliest flavoprotein oxidases discovered. This enzyme [EC 1.4.3.3, L-amino-acid:oxygen oxidoreductase (deaminating)] is a dimeric protein, with a subunit molecular weight of 38 kDa and containing a molecule of FAD per subunit [70]. DAAO principally is localized in the peroxisomal compartment of the pig kidney and is closely related to D-aspartic acid oxidases, comprising a small family of proteins with similar function. DAAO metabolizes most D-amino acids containing a single carboxylic acid group, from D-alanine to D-tryptophan, while D-aspartic acid oxidases metabolize dicarboxylic amino acids. The enzymes require substrates with both an amino and carboxylic acid group to achieve high levels of oxidative metabolism, and

the keto acids of the amino acids formed during oxidative metabolism by this enzyme are product inhibitors of the reaction. Since D-amino acids cannot be used for protein synthesis, the existence of DAAO in kidney lysosomes is believed to be critical for the disposition of D-amino acids.

12.3.2.3 L-Amino Acid Oxidases (LAAO). The substrate specificity for L-amino acid oxidase (LAAO), L-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2, includes the L-amino acids, namely, alanine, aspartate, methionine, valine, leucine, isoleucine, tyrosine, phenylalanine, and tryptophan. Similar to DAAO, the keto acid metabolites of these amino acids are also inhibitors of this enzyme system. The enzyme purified from rat kidney was shown to have a subunit molecular weight of 49.3 kDa determined by flavin content, and on the basis of this molecular weight and the amount of flavin associated with the protein, is consistent with two FAD molecules per molecule of enzyme [71]. It has been assumed that this enzyme allows the clearance of L-amino acids that accumulate in the kidney because of excessive protein catabolism and release from various tissues before excretion. In murine lymphocytes, this enzyme was shown to have a molecular weight of ~90 kDa, suggesting its existence as a dimer in this cell type [72]. The enzyme's function in lymphocytes has been suggested to be antigen processing in lysozomes. During the development of drugs, namely, levodopa and carbodopa, that protect against excessive dopamine metabolism in brain, researchers were concerned that LAAO might be involved in the metabolism of dopamine and therefore may be a drug target for inhibition or inactivation. Other LAAOs in nature have been purified from venoms of various vipers, that is, *Calloselasma rhodostoma, Naja naja kaouthia*, and *Ophiophagus hannah*. These enzymes have similar properties to those characterized in mammalian species.

12.3.2.4 Polyamine Oxidases. Another member of the flavin amine oxidase family are enzymes that oxidize polyamines (EC 1.5.3.11). These enzymes are highly conserved among humans, mice, and rats and act on the acetylated products of spermine and spermidine, namely, N^1-acetylspermine and N^1-acetylspermidine, to produce spermidine and putrescine as products. N^1, N^{12}-diacetylspermine, formed by acetylation of spermine via spermidine/spermine N^1-acetyltransferase, is also a substrate for the enzyme. The molecular weight of the polyamine oxidases is 61.8 kDa, and they contain FAD as the prosthetic group [73,74]. These enzymes are believed to be critical in maintaining polyamine levels in cells, although in at least some cells, the coupling of the polyamine transporter, SLC3A2, to the spermidine/spermine N^1-acetyltransferase suggests an equally important mechanism to control intracellular polyamine levels [75].

12.4 AZO REDUCTASES

Another set of reactions that have not been as well characterized as the dealkylation or ring hydroxylation reactions of cytochrome P450 are the azo, nitro, and other reduction reactions [2]. The azo reduction reactions have been of interest to pharmacologists and toxicologists for some time because of their effect on the azo dye industry, which produces azo dyes that are largely used for cloth coloration or as pharmaceutical agents for preventing bacterial contamination. In his 1991 review, Levine [76] described the role of azo dyes in carcinogenesis and mutagenesis and also addressed reductive

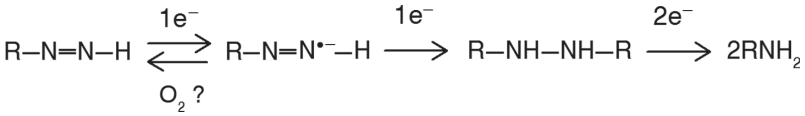

Figure 12.2 Steps of azo compound reduction to hydrazine and primary amines, including the putative reoxidation of radical intermediates by molecular oxygen. Hydrazine compounds have also been shown to be oxidized by cytochrome P450s, copper-containing amine oxidases, and monoamine oxidases [4–7].

metabolism in bacterial and mammalian cells. The oxidative metabolism of azo dyes includes N-dealkylation, aromatic ring hydroxylation, and N-hydroxylation to form azoxy derivatives, which have been extensively studied [68,77,78]. All these reactions tend to be catalyzed by the cytochrome P450s, and in the case of procarbazine, the oxidation of this 1,2-dialkylhydrazines to the azoxy derivatives leads to formation of a reactive methyldiazonium ion that probably accounts for the alkylation potential of this anticancer drug [7,79]. Azo functional group reduction occurs in the absence of molecular oxygen, and depending on the azo compound in question, reduction can occur in the presence of molecular oxygen. Since cytochrome P450 requires molecular oxygen for oxidation of C- and N-centers, only the reduction reaction can occur in the absence of oxygen. The reaction steps for azo reduction are shown in Fig. 12.2.

This review focuses on the unique azo reduction reactions catalyzed by mammalian liver microsomes and some bacterial systems. Interest in azo reduction was increased after the unique work of Bovet and others [80,81], who elucidated the need for reductive bioactivation of the antibacterial azo dye protosil, which on reduction yields sulfanilamide in rabbits. This is a classic prodrug reductive activation process. Other azo compounds were subsequently developed as effective antibacterial agents, but often, their azo linkage was not reduced, leading to excretion through a glucuronidation pathway. Miller and Miller [82] who studied the metabolic activation of butter yellow (*N*, *N*-dimethylaminoazobenzene) also demonstrated that NADPH in the presence of liver microsomes and oxygen could not only sustain the demethylation of this azo dye but also reduce it to *N,N*-dimethyl-*p*-phenylene-diamine [77,78] The chemical properties of various azo dyes appear to dictate how easily they are reduced; these properties are addressed in the following sections as well. In addition to the cytochrome P450s, other enzymes play a role in azo dye reduction, principally flavoproteins.

12.4.1 Flavoproteins

As mentioned by Levine in his 1991 review [76], many azo dyes used in agricultural or clinical applications are taken orally, and only a small fraction of the parent compound is excreted in the urine with the azo linkage intact, suggesting that reductive metabolism is a major route of disposition. If administered by routes other than per os, substantial biliary excretion of the intact dye can be observed in experimental animals. The lack of reduced azo compound in bile suggests that if azo reduction occurs, the metabolites are rapidly oxidized back to the azo compound in liver or that the gut microflora may be significantly involved in disposition of azo dyes. Their uptake is dependent on solubility because those with charged groups, such as sulfonates, are apparently not well absorbed. More lipid-soluble dyes, such as dimethylaminoazobenzene, were shown

by the Millers [77,78] to be extensively metabolized by both oxidative and reductive metabolism when administered by intraperitoneal injection. Their work demonstrated step-wise N-demethylation and ring hydroxylation reactions leading to products that are excreted into the bile as glucuronides, while reduction to stable products such as amine compounds leads to urinary elimination.

Walker published a review on azo compound metabolism in 1970 [83] and summarized for the first time the understanding of azo dye metabolism. Several important points were made in this review, including the fact that several groups had shown that water-soluble azo dyes were mostly reduced in the gut, based on the pronounced effects of antibiotics. Ryan and Wright [84] used the dye tartrazine, which was not reduced by liver extracts in the presence of NADPH. When added to cell-free extracts of *Proteus vulgaris*, several azo dyes were shown to be rapidly reduced, supporting the view that bacterial cells can reduce azo dyes. In subsequent work, Ryan and coworkers [85] showed that a number of bacterial isolates of differing origin can reduce azo dyes, and thereby, they established that gut bacteria are an important source of reduction in rodents and probably humans. At this time, others were purifying the bacterial NAD(P)H-dependent reductases and several enzymes were shown to reduce a number of dyes [86,87]. The bacterial flavoproteins normally do not bind FAD or FMN as tightly as the mammalian flavoproteins do, so the addition of FAD or FMN was required to optimize the azo reduction reaction.

Hernandez *et al*. [88,89] noted that purified lipase-solubilized NADPH:POR catalyzed the anaerobic reduction of the azo compound neoprotosil to sulfanilamide. Using inhibitors of the oxidoreductase, the microsomal azo reductase activity was concordantly inhibited with the activity of the purified enzyme. Studies at the NIH by Gillette's group also addressed nitro reduction reactions, and the group found that these reactions were stimulated by the addition of FAD. Similar experiments with exogenous FAD were performed by Walker [83], where he noted that if FAD was added to liver microsomal fractions, the azo reductase activity increased. The apparent azo substrate specificity approximates first-order kinetics, because of the slow step of reduction being the reaction of reduced flavin with the dye. Many bacterial flavoproteins have since been isolated and nearly all reduce azo and nitro compounds, suggesting that this reaction is a nonphysiological reaction of nearly all flavoproteins [86,87,90]. These reactions most likely account for the metabolism of azo dyes in the intestine (Eqs. 12.8–12.10), although flavin-independent reactions can occur with bacterial enzyme systems by direct reduction with reduced pyridine nucleotides [91]. However, many of these flavoprotein-catalyzed reductions are oxygen sensitive and the reduction reactions are normally only seen during anaerobic metabolism, depending on the dye substrate's chemical properties (electrochemical potential, etc.).

$$\text{E–FAD} + \text{NAD(P)H} + \text{H}^+ \longrightarrow \text{E–FADH}_2 + \text{NADP}^+ \tag{12.8}$$

$$\text{E–FADH}_2 + \text{FAD} \longrightarrow \text{E–FAD} + \text{FADH}_2 \tag{12.9}$$

$$2\text{FADH}_2 + \text{R–N=N–R} \longrightarrow 2\text{FAD} + 2\text{RNH}_2 \tag{12.10}$$

12.4.2 Cytochrome P450

The studies of Mueller and Miller [77,78] were among the first to address the possibility of cytochrome P450 serving as the reducing enzyme for azo reduction. Masters

et al. [92,93] had shown that steapsin, a pancreatic lipase preparation from pigs, could release a cytochrome *c* reductase enzyme activity from liver microsomes. Masters' group subsequently purified the membrane-bound form of the flavoprotein [94], which retains its azo reductase activity (Masters BSS, Prough RA, unpublished research). Their subsequent work demonstrated that the enzyme released by lipase treatment was a proteolytic form of the NADPH:POR. Hernandez *et al*. [88,89] used the method of Masters *et al*. [92] to purify the oxidoreductase to homogeneity and demonstrate that the pure enzyme could reduce neoprontosil under anaerobic conditions. When Hernandez *et al*. [88,89] utilized steapsin to release the oxidoreductase from the microsomal membrane, they noted that azo reductase activity was decreased to 25% of control, but NADPH:cytochrome *c* reductase activity actually increased 20%; neotetrazolium dye reduction to formazin also increased slightly. They also noted that on induction of cytochrome P450 in liver microsomes by 3-methylcholanthrene, the microsomal azo reductase activity increased without a concordant increase in reductase activity, suggesting that the polycyclic aromatic hydrocarbon inducible cytochrome P450s (the CYP1 family) were azo reductases. When they used phenobarbital as an inducing agent, both cytochrome P450 and oxidoreductase levels were induced. Subsequently, they used carbon monoxide as an inhibitor of cytochrome P450-dependent reactions and noted that CO decreased microsomal azo reductase activity by 30–40% with liver microsomes from control, 3-methylcholanthrene- or phenobarbital-treated rats. The azo reductase activity of purified oxidoreductase was unaffected by replacing the air atmosphere with CO. Hernandez *et al*. [88,89] proposed that there were two enzyme systems that resulted in anaerobic reduction of neoprontosil, the first was the NADPH:POR itself and the other system was cytochrome P450, which itself requires two-electron transfer from the oxidoreductase to maintain its catalytic process (Fig. 12.2).

The subsequent work of Levine *et al*. [76,95,96] addressed several points about azo dye reduction; first, which cytochrome P450s catalyze azo reduction and second, why some azo dyes required anaerobiosis for reduction by these systems and others do not. In their studies, they identified two types of azo dye substrates related to 4-dimethylaminoazobenzene, those substrates whose reduction was sensitive to O_2 and CO and those that were insensitive [95,97]. A common chemical feature of the two classes was that the insensitive class tended to have electron-donating ring substituents such as -OH, -NH_2, -NH-CH_3, or -$N(CH_3)_2$ group. The sensitive class of substrates contained electron-withdrawing groups, such as -SO_3H, -COOH, -$COOCH_3$, and -AsO_3H_2. They also noted that cyanide ion potently inhibited the reduction of the O_2-insensitive azo dye substrates, suggesting that the insensitivity/sensitivity may be a property of the ferrous cytochrome P450 [98]. Azide did not differentially affect the azo reduction of their set of compounds. One explanation for the differences in O_2/CO sensitivity was that the hydrazine intermediates (Fig. 12.2) may have differential sensitivity to reoxidation by molecular oxygen to the azo intermediate or alternatively be reduced to the primary amine metabolite. On the basis of the work from our groups, we know that hydrazines are easily oxidized by cytochrome P450s and are also sensitive to metal-catalyzed oxidation [68,99]. The mechanism accounting for the oxygen sensitivity or insensitivity remains unsolved.

Pursuing the approach of Hernandez *et al*. [88,89], Levine demonstrated that induction of liver microsomal enzymes from animals treated with various chemicals greatly affected azo dye reduction and each condition displayed a different substrate specificity for azo dyes [100]. Induction by clofibrate increased azo dye reductase activity

for nearly all dyes, suggesting a role for the CYP4 family of cytochrome P450s in azo dye reduction, while phenobarbital, pregnenolone-16α-carbonitrile, and isosafrole were all inducers of azo dye reduction for *O*-methyl red [8,100]. Their results demonstrated that members of the CYP1, CYP2, CYP3, and CYP4 families all reduce azo dyes under anaerobic conditions and depending on the structure of the azo dye, possibly under aerobic conditions. Mallett *et al*. [101,102] utilized purified NADPH:POR and CYP2B1 to reconstitute the anaerobic reduction of the azo dye amaranth, an oxygen-sensitive reaction. In addition, they added FMN and FAD to yield higher rates of reduction of the dye. In the absence of FMN, amaranth reduction was dependent on oxidoreductase, CYP2B1, and dilauroylphosphatidylcholine; in the presence of FMN, the oxidoreductase appeared to be the source of reduction. They proposed two mechanisms of reduction for amaranth: direct reduction by oxidoreductase that was stimulated by addition of FAD and cytochrome P450-mediated reduction in the absence of FAD. Clearly, CO-insensitive azo reduction reactions are apparently unaffected by flavin deficiency, while the flavoprotein-dependent azo reduction reactions are heavily affected by depletion of flavin *in vivo* or supplementation of flavin *in vitro* [103].

Milton *et al*. [104] demonstrated that clofibrate was a potent inducer of CYP4A1 in rat liver and defined several inhibitors of the enzyme, including saturated fatty acids and clofibrate. Raza and Levine [105,106] demonstrated that azo dyes were most effectively reduced in liver microsomes from ciprofibrate-treated rats. In addition, they demonstrated that addition of *N,N*-dimethyl-4-aminoazobenzene to reaction mixtures containing liver microsomes from clofibric acid-treated rats inhibited lauric acid hydroxylation and that lauric acid inhibited *N,N*-dimethyl-4-aminoazobenzene reduction with these same microsomal fractions. Their work also demonstrated that a CYP4A-specific inhibitor, 10-undecynoic acid, inactivates laurate hydroxylation, but not azo reductase activity, suggesting that the active site for oxidative metabolism is affected by the inhibitor for laurate oxidation, but not that for azo dye reduction. In addition, they reconstituted NADPH:POR with purified CYP4A1 to demonstrate that *N,N*-dimethyl-4-aminoazobenzene azoreduction is catalyzed by this enzyme in the absence or presence of oxygen, but in the presence of oxidoreductase alone, the flavoprotein could only reduce it under anaerobic conditions. Finally, they noted that in the reconstituted system, inclusion of FAD greatly enhanced azo reduction; the oxidoreductase alone was nearly as effective in reducing azo dyes in the presence of FAD than the reconstituted system. Lu and Levine [8] performed a more detailed study that demonstrated that azo reduction by cytochrome P450 is a two-electron process yielding the stable hydrazine (two-electron) or amine (four-electron) reduction product, while the oxidoreductase apparently only catalyzes one-electron reduction to an intermediate that is rapidly oxidized to the azo derivative.

Walker [83] and Levine [76] discussed the one- and two-electron reduction processes involved in azo dye reduction, arguing that those that are oxygen-insensitive must form stable two-electron reduced products like the hydrazine derivative, while oxygen-sensitive processes may catalyze one-electron reduction to a radical. It is known that molybdopterin-containing enzymes [76], such as aldehyde and xanthine oxidase, also reduce azo dyes but are sensitive to cytochrome P450 inhibitors, such as SKF-525A and metyrapone. These complex redox centers have common properties to the cytochrome P450s that make defining specific inhibitors difficult. However, one should consider whether any reduction reactions are noted to be oxygen/CO sensitive when studying

new molecules that are reduced, as well as the relative sensitivity to cyanide and azide ions to characterize various reactions.

12.5 NITRO REDUCTASES

Similar studies have been performed with nitro compounds, and among the early works, Gillette's group at the NIH documented that anaerobic nitro reduction of *p*-nitrobenzoate was catalyzed by liver microsomal protein in the presence of NADPH [107,108]. Carbon monoxide atmosphere caused an 80–90% inhibition of nitro reduction. In addition, they studied the formation of aniline from nitrobenzene, nitrosobenzene, and phenylhydroxylamine, noting that nitrosobenzene and phenylhydroxylamine were rapidly converted to aniline in a CO-sensitive reaction. The oxygen sensitivity of aniline production from any of the proposed metabolites reported in the literature suggests that anaerobiosis is required for net reduction due to the ease of oxidation of the substrate and intermediates by oxygen. On the basis of their studies, they described a reaction that required four 1-electron reduction steps (Fig. 12.3).

Other groups studied the role of NADPH:POR in these processes. For example, Delaforge *et al*. [109] demonstrated that trinitroglycerin is also reduced to eventually allow liberation of nitric oxide. Aristolochic acid is a nutraceutical found in evergreen and deciduous woody vines and herbaceous perennials of *Aristolochia* and *Asarum* of the Aristolochiaceae plant subfamily and is a naturally occurring nephrotoxic nitro compound. On reductive bioactivation, it forms DNA adducts in rodent tissues *in vivo* [110]. Bioreductive activation of the 6-nitro group of aristolochic acid by members of the CYP1A family has been proposed by Stiborova *et al*. [110] as the bioreductive activation mechanism of this chemical. Reduction of the nitro group leads to formation of a putative nitrenium intermediate and its subsequent reaction with nucleic acid bases results in formation of DNA and protein adducts.

Harada and Omura [111] provided mechanistic details about nitro reduction by demonstrating that inhibitory antibodies to NADPH:POR and several cytochrome P450s inhibited anaerobic reduction of *p*-nitrobenzene, but not antibodies against NADH:cytochrome b_5 oxidoreductase or cytochrome b_5. They also reconstituted the purified oxidoreductase and cytochrome P450s to document that the latter were required for the final step of reduction of phenylhydroxylamine to aniline, while the oxidoreductase could catalyze reduction of nitrobenzene and nitrosobenzene alone in the presence of NADPH. Moreno *et al*. [112] utilized electron spin resonance (ESR) to study trapped radical species formed during *p*-nitrobenzene reduction. They observed that under aerobic conditions, the major radical trapped was superoxide

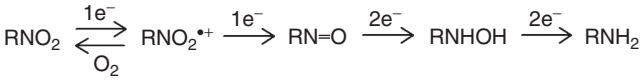

Figure 12.3 Steps of nitro reduction to nitrosoamine, hydroxylamine, and primary amine derivatives, including the putative reoxidation of radical intermediates by molecular oxygen. Hydroxylamine compounds have been shown to be oxidized to nitroso derivatives by cytochrome P450s [6].

anion radical. At low oxygen concentrations, the putative radical species undergo intramolecular electron transfer and rearrange to form carbon-centered nitrobenzyl radicals that could also be measured by ESR. Radical trapping was not inhibited by CO or the nitrogenous cytochrome P450 inhibitor metyrapone. Radical formation for *m*-nitrobenzyl anion radical did not achieve a steady-state concentration, unless metyrapone or CO was included in the reaction mixture. This observation suggests that the POR is a major source of reduction [113,114], and this oxidoreductase-dependent reaction is increased by inclusion of cytochrome P450 inhibitors.

The study of other nitro compounds, 4-nitroquinoline *N*-oxide and 4-hydroxylaminoquinoline *N*-oxide, strongly supports the role proposed for the oxidoreductase in their reduction [115]. Furthermore, the studies suggest that this reaction is due largely to one-electron reducing flavoproteins such as POR since two-electron-donating flavoproteins, such as NAD(P)H:quinone oxidoreductase, do not generate superoxide anion radical. However, POR can reduce the one-electron reduced nitro compounds further, unless molecular oxygen is present to oxidize the radical species [114]. Many bacterial flavoproteins in the gut also catalyze these reactions and may protect mammals from nitro compounds taken in the diet. Bacterial and mammalian flavoproteins appear to be particularly effective in catalyzing reduction of nitro compounds, and the role of cytochrome P450s is perhaps less important for these compounds.

12.6 OTHER ENZYME-CATALYZED REDUCTION REACTIONS

A number of other reports of substrates being reduced by flavoproteins and possibly by cytochrome P450s have been reported. These have not been studied in depth, and anecdotes of such reports are included in the discussion below.

12.6.1 Quinones

Quinones have long been known to be reduced by most flavoproteins. The studies of Masters *et al*. [92] show that POR reduces cytochrome *c*, menadione, and 2,6-dichlorophenol indophenol. Our laboratories [116–118] also have studied the reduction of *t*-butylquinone by both POR and NAD(P)H:quinone oxidoreductase, suggesting that benzocyclic, napthocyclic, and complex polycyclic aromatic hydrocarbon quinones are good electron acceptors for the flavoproteins. POR serves as a one-electron reducing flavoprotein, while NAD(P)H:quinone oxidoreductase serves as a two-electron reducing flavoprotein. In the case of one-electron reduced semiquinone radicals, they rapidly react with molecular oxygen to generate superoxide anion radical, but two-electron reduced hydroquinones react more slowly with molecular oxygen in the presence of metal catalysts [116,119]. A consequence for this difference is that one attains higher levels of quinone reduction by two-electron reduction because less radical quinone intermediate is available for reoxidation by molecular oxygen (Fig. 12.4). For the purpose of this review, we address only information known about reactions catalyzed by cytochrome P450s.

Capdevila *et al*. [120] used spectroscopic methods to study quinone reduction and reoxidation of benzo[*a*]pyrene-3,6-quinone as a model quinone. Reduction and reoxidation could be monitored at 480 nm (wavelength maxima for B[*a*]P-3,6-quinone). The quinone was observed to be rapidly reduced by liver microsomal fractions. The

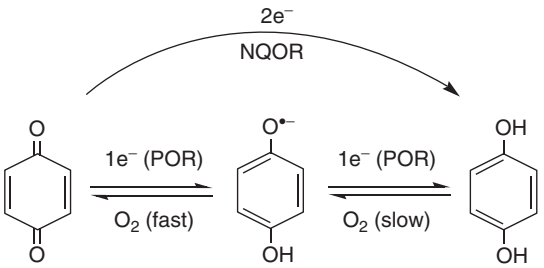

Figure 12.4 Reduction of quinones by the flavoproteins, NADPH:cytochrome P450 oxidoreductase (POR), or NAD(P)H:quinone oxidoreductase. Semiquinone radicals reoxidize rapidly in the presence of molecular oxygen to yield the parent quinone and superoxide anion radical, while hydroquinones react less well with molecular oxygen [119].

reoxidation process was coupled to molecular oxygen reduction and hydrogen peroxide formation. The initial rates of oxidation were inhibited by anti-POR globulin and the nitrogenous ligand, metyrapone. Of interest, anaerobiosis or CO had no affect on the initial rates of reduction, only reoxidation was prevented. During steady state of the reaction, the quinone exists largely as the hydroquinone, but the level of reduction increases under anaerobic conditions, suggesting a redox cycling phenomenon similar to that seen with azo dyes and nitro compounds under aerobic conditions. This reaction was partially supported by purified POR but was not inhibited by metyrapone or CO [120]. The reoxidation reaction indicated that insufficient reduction occurred to attain full steady-state reduction of the quinone as seen with NAD(P)H:quinone oxidoreductase, known to catalyze two-electron reduction of menadione and *t*-butylquinone. Alternatively, the B[*a*]P quinone may be further metabolized by oxidative metabolism forming more polar metabolites. With benzyl- and naphthylquinones, total reduction of menadione and *t*-butylquinone can be achieved with purified NAD(P)H:quinone oxidoreductase, but not with purified POR [119,120]. These studies suggested one-electron reduction processes are involved in reduction involving both POR and cytochrome P450s, but not NAD(P)H:quinone oxidoreductase (Fig. 12.4). Complete reduction was never achieved in the presence of oxygen with either flavoprotein because of the slow comproportionation equilibrium of the quinone/hydroquinone couple (Eq. 12.11).

$$QH_2 + Q \longrightarrow 2QH \tag{12.11}$$

Vermeulen and coworkers [121,122] also noted that 2,3,5,6-tetramethylbenzoquinone (TMQ) was reduced to a one-electron reduced quinone in the presence of CYP2B. This reaction was apparently inhibited by SKF-525A, another nitrogenous ligand inhibitor of cytochrome P450. In a reconstituted enzyme system of POR and cytochrome P450, they followed the reduction of TMQ by ESR and noted that the semiquinone radical was produced at a higher rate when cytochrome P450 was reconstituted with POR than POR did itself. Cytochrome P450 inhibitors and inhibitory antibodies also were effective in inhibiting TMQ reduction. They concluded that reconstitution of TMQ reduction by POR with increasing concentrations of cytochrome P450 was correlated with the level of the hemoprotein in the reactions, thereby proving the role of cytochrome P450 as a reductive catalyst. Subsequent work

from this group on adriamycin and mitomycin C documented roles for P450 2B1 in one-electron reduction of these quinone compounds and their bioactivation by this process [122–124].

12.6.2 Reductive Dechlorination

Guengerich [2,125,126] has provided a number of examples from the literature for reductive dechlorination reactions. Other reviews have also summarized these reduction reactions [127,128]. The best studied dechlorination reactions are those monitoring metabolism of carbon tetrachloride [129] and halothane under anaerobic conditions [130–132]. These reduction reactions principally form reduced products in which the halide group is replaced by hydrogen or by the formation of olefins (Fig. 12.5). In each case, cytochrome P450 provides an electron to the parent halide compound from its ferrous iron species, liberating a halide ion and a carbon-centered radical. The chemistry of the radical appears to determine the product formed. For example, the products can react with molecular oxygen to form an oxidized product, as seen with the trichloromethane radical produced from $CCl_3^{\cdot}$ radical. Interaction with molecular oxygen allows production of phosgene. Alternatively, loss of a second halide ion from the radical allows formation of an olefin, as seen during the reduction of halothane. Both reactions led to the formation of reactive intermediates capable of forming adducts with nucleophiles such as protein and nucleic acid bases [130–132]. As pointed out by Guengerich [2], Castro and Castro [133] proposed a consistent mechanism for reductive dehalogenation reactions (Fig. 12.5).

12.6.3 Reduction of Hydroperoxides

Hydroperoxides are also reduced by cytochrome P450s to yield alcohol and water (Eq. 12.12), as discussed by Guengerich [2]. The mechanism of this reaction is not fully appreciated since organic hydroperoxides apparently function as alternate donors of an atom of oxygen to ferric cytochrome P450 to form a perferryl species, a reaction similar to the key step in the P450 catalytic cycle involved in heterolytic cleavage of molecular oxygen [134]. Many examples of these hydroperoxide reduction reactions exist in the literature for the prostaglandin synthetases, lipoxygenases, and cytochrome P450s, in which a lipid hydroperoxide is reduced to a lipid alcohol [135]. Cytochrome P450s can not only produce lipid peroxides during the course of catalysis but can

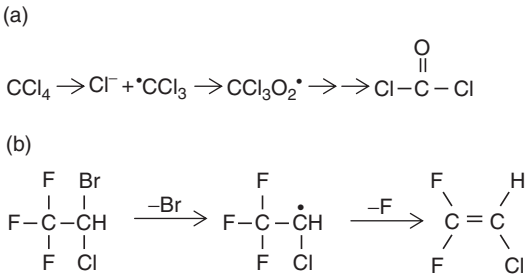

Figure 12.5 Steps of reductive dehalogenation reactions: (a) molecular oxygen reacts with a radical (halide) to lose halogen [133] and (b) reductive dehalogenation forms olefins [129].

also convert such intermediates to the hydroxyeicosenoic acid as part of their catalytic mechanism.

$$RCH_2O{-}OH + Fe^{III} \longrightarrow RCH_2{-}OH + Fe^{V}{-}O \qquad (12.12)$$

12.6.4 α,β-Unsaturated Aldehydes

Recently, we have studied the metabolism of α,β-unsaturated aldehydes derived from lipid peroxidation, such as 4-hydroxy-2-nonenal and acrolein, using primary hepatocytes and expressed cytochrome P450s. We have shown that many cytochrome P450s catalyze the oxidative metabolism in an NADPH- and O_2-dependent manner [136]. Specific cytochrome P450s of the CYP3A, 2B, and 2 C families were effective in oxidizing aldehydes to their carboxylic acid. In recent studies [137], we also have detected a reaction in which the 4-hydroxy-2-nonenal is reduced to 4-hydroxy-2-nonenol in the presence or absence of oxygen. The reaction was not affected by replacing the normal air atmosphere with carbon monoxide or addition of inhibitors, such as metyrapone, to block the reaction. The α,β-unsaturated bond in aldehydes may allow reduction of these aldehydes to their alcohols, much like the oxygen-insensitive azo reduction reactions. This precedent suggests that there may be other reduction reactions of olefinic compounds by cytochrome P450s, and the tools for the study described by Levine and others may be important approaches to characterize these reactions.

12.6.5 Summary of Cytochrome P450 Reduction Reactions

Except for our report of 4-hydroxynonenal reduction, no cytochrome P450-dependent reduction reactions for endogenous compounds are known. As pointed out by Guengerich in his 2001 review [2], it is peculiar that this heme protein catalyzes reduction reactions at all because of the high reactivity of the hemoprotein in its ferrous form with molecular oxygen. The scientific community assumes that all ferric cytochrome P450s bind oxygen and subsequently the molecular oxygen is rapidly reduced. In tissues with low oxygen tension, reduction reactions might be favored [138]. Because the oxidation–reduction potential for ferric cytochrome P450s is around −300 mV, in the absence or presence of substrate, this species of cytochrome P450 should be an excellent one-electron reductant. Further study of the chemical mechanism of the cytochrome P450s is needed to address this anomaly. However, discovery of a reduced metabolite might direct one to assume either a flavoprotein or cytochrome P450 may be involved in these reactions.

12.7 CONCLUSIONS/FURTHER PERSPECTIVES

In the past, considerable attention has been directed toward the role of cytochrome P450 and FAD-containing oxygenase in amine oxidation reactions. However, an increasing number of studies in the literature require researchers to rediscover the role of the quinone- and flavin-dependent amine oxidases. This chapter attempts to describe the enzyme systems that can catalyze these reactions and provides examples of the reactions with exogenous compounds that have been shown to involve these enzymes. In addition, this chapter addresses the rare reduction reactions of flavoproteins and the

cytochrome P450s as a guide to the reader for considering rare reactions that occur in drug metabolism. The study of metabolic enzymes has been extensive, but it is clear there is much to be learned about these proteins on a single gene/protein level. Because bioreductive reactions for foreign compounds are rare, the authors hope that this review has provided a road map to assist in identification of such systems.

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